ARE THE CONTENTS OF EGG CAPSULES OF THE MARINE GASTROPOD *NUCELLA LAPILLUS* (L.) AXENIC?

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ABSTRACT

The fluid from egg capsules of *Nucella lapillus* was found to be axenic when capsules contained living embryos. One hundred percent of excapsulated, pre-shelled embryos survived and developed for 21 days in sterile seawater to which antibiotics were added, while control embryos in unsterile, 0.45 μm filtered seawater died after four days. Providing early embryos with protection from bacteria may be one role for egg capsules. Since embryos could survive and develop outside capsules, the capsular fluid may not be necessary for growth of embryos of this species.

Thorson (1950) suggested that the fluid of gastropod egg capsules may have bacteriostatic properties, but subsequent studies on the fluid from capsules of four species (*Searlesia dira* [Reeve], *Nucella* [= Thais] *lamellosa* [Gmelin], *N. lima* [Gmelin]; Rivest, 1981; *N. lapillus* [L.]; Pechenik *et al.*, 1984) provided no evidence that the fluid deterred bacterial growth. However, if an egg capsule were impermeable to bacteria, and if the contents of that capsule were axenic when the capsule was formed, then an egg capsule could provide a bacteria-free environment for developing gastropod embryos, even though capsular fluid is not bacteriostatic. Recent studies have shown that eggs and sperm of the oyster, *Crassostrea gigas* (Thunberg) (Langdon, 1983) and the purple sea urchin, *Strongylocentrotus purpuratus* (Stimpson) (Manaham *et al.*, 1983) are axenic before discharge from the gonads. If the reproductive tracts of gastropods that make egg capsules are bacteria-free, then these gastropods could produce capsules with axenic contents.

The multilayered, vase-shaped egg capsule of the dog whelk, *Nucella lapillus*, has an outer layer of mucopolysaccharide, and the capsule wall is composed of a conchiolin-like material made of protein associated with polysaccharide (Bayne, 1968). Pechenik (1983) found that the tough capsule wall of this species is permeable to NaCl and water, less permeable to amino acids, glucose, and sucrose, and appears to be non-permeable to large organic molecules (proteins and neutral polysaccharides; Bayne, 1968) found in the capsular fluid. If the capsule wall is impermeable to large molecules, then it is unlikely to be permeable to bacteria. Even small marine bacteria (0.5 μm in diameter; Hobbie *et al.*, 1977) are 150 times wider than the average globular protein.

Egg capsules of *N. lapillus* contain about 1.1 μl of fluid per embryo, and an average of 33.7 $\pm$ 16.3 embryos per capsule (Pechenik *et al.*, 1984). Packaged with the eggs that will develop into embryos are nurse eggs, on which the embryos feed during the first week of their development (Costello and Henley, 1971). After they have consumed the nurse eggs, the embryos resemble unshelled, yolk-filled sacs.

Pechenik *et al.*, (1984) attempted to rear both pre-shelled and shelled excapsulated embryos. Shelled embryos were reared in 0.45 μm filtered seawater for 29 days with 28% mortality, but 94.7% of the pre-shelled embryos died in 18 days. Pechenik *et al.*, (1984) did not determine whether bacterial contamination affected mortality of the pre-shelled embryos.

In this study I examined fluid and embryos from egg capsules of *N. lapillus* to determine whether the contents are bacteria-free and have raised pre-shelled, excapsulated embryos in autoclaved seawater with antibiotics to determine the influence of a bacteria-free environment on survival of the early embryos. Individuals are considered to be embryos until they escape from the egg capsule (Giese and Pearse, 1974). Embryos of *N. lapillus* hatch as crawl-away juveniles.

MATERIALS AND METHODS

Intertidal egg capsules of the prosobranch gastropod *Nucella lapillus* were collected from Nahant, Massachusetts during May and July, 1985, and kept at 14-16°C in seawater filtered to 1 μm. Water was changed every other day.

To determine whether the capsular fluid of *N. lapillus* is axenic, fluid was removed from capsules and incubated overnight at room temperature in 5 ml of nutrient broth (0.20 μm filtered seawater, 0.25% yeast extract, and 1% peptone; Pechenik *et al.*, 1984). Presence of bacteria in the nutrient

broth was determined by inspection. If no bacteria are present, the broth remains clear; contaminated broth becomes turbid and a thick scum of bacteria forms on the surface of the fluid within 24 hours.

Before fluid was removed, capsules were dipped in 95% ethanol to reduce bacterial contamination on the outer capsule surface. Dipping in 95% ethanol eliminates growth of surface bacteria for 24-36 hours. The fluid of newly deposited capsules is viscous (Pechenik, 1983) and clogs narrow gauge needles; a 21 gauge needle was therefore used to remove contents of newly deposited capsules. The fluid becomes non-viscous about five days after capsule deposition (Pechenik, 1983) and a 25 or 30 gauge needle was then used to suck out fluid while leaving embryos intact. After fluid was removed, capsules were cut open and the number of embryos per capsule and their developmental stage were noted.

Although it is unlikely that capsular fluid would be contaminated while embryos were axenic (or *vice versa*), it is possible that the techniques used to remove the fluid could contaminate capsule contents or kill bacteria in it. Therefore, embryos were also tested for contamination as a control. After being dipped in 95% ethanol, capsules were cut open and embryos were emptied into 0.2 μm filtered, autoclaved seawater. Embryos were added to the broth and incubated overnight at room temperature. Aliquots of water into which capsule contents had been emptied were checked before and after embryos were added to be sure water was sterile.

Fluid from capsules containing dead embryos was also checked for bacterial contamination. Capsules containing dead embryos can be recognized because when embryos of the genus *Nucella* die, they generally turn a purplish-pink color visible through the capsule wall (Spight, 1975; Gallardo, 1979; Pechenik, 1982, 1983). The fluid from capsules containing embryos dead at the time of collection, and from capsules in which embryos were killed by keeping the capsules overnight in deionized water, was examined for bacterial contamination as described above. Embryos from capsules kept in deionized water turned pink during exposure. Dead embryos were also tested for contamination.

To ensure that overnight exposure to deionized water did not kill bacteria, controls in which bacteria from the surface of capsules were cultured and then exposed to deionized water were run. After overnight exposure to deionized water, bacteria were added to culture broth, and the broth was checked after 24 hours.

To determine whether pre-shelled, excapsulated embryos could be raised in bacteria-free seawater, I passed seawater through a 0.20 μm Schleicher and Schuell filter, autoclaved the filtrate, and added the antibiotics penicillin (40 mg/l) and streptomycin (50 mg/l). Embryos were removed from five capsules by clipping off the capsule tops and emptying the capsule contents into sterile seawater. Eight embryos plus a portion of the nurse egg mass with embryos attached were placed in each of three replicate dishes containing 15 ml of the treated seawater. As a control, eight embryos were added to a dish of 0.45 μm filtered seawater that was not autoclaved and to which no antibiotics were added. Embryos were kept at 14°C for up to 21 days and checked daily for mortality and development. Water was changed daily, and Day 1 was the day of excapsulation.

The fluid from egg capsules of two other gastropod species, *Buccinum undatum* (L.) (3 capsules) and *Thais haemastoma canaliculata* (Gray) (4 capsules) was also examined for bacterial contamination using techniques described above. *Buccinum undatum* capsules were collected from the walls of seawater tables at Northeastern University's marine lab, Nahant, Massachusetts. At the time fluid was sampled, embryos were still yolky and undeveloped, and fluid was slightly viscous. *Thasis haemastoma canaliculata* capsules were collected by Dr. C. D'Asaro in Florida and shipped to Massachusetts in late May. Two of the four capsules examined were a clear, creamy color and contained shelled embryos. Two capsules were darker brown, indicating that capsules were older and embryos were ready to emerge (R. Dobberteen, pers. comm.).

Capsular fluid and embryos were manipulated using sterile glassware in a sterile hood.

RESULTS

Fluid from capsules of *N. lapillus* containing living embryos was axenic in all cases examined. Of the 17 capsules containing pre-shelled to fully shelled embryos, none had fluid containing bacteria that grew in the nutrient broth. However, of 13 capsules containing dead embryos, the fluid within five capsules contained bacteria that grew overnight in the broth. None of the capsules exposed to deionized water contained bacteria, although bacterial contamination was found in fluid from field-killed capsules in which embryos were dead but not pink. Bacteria exposed to deionized water grew normally after being returned to broth and formed a scum on the broth surface within 24 hours.

Living embryos from three capsules were axenic, and the water into which the capsules were emptied was sterile. Dead embryos from one capsule out of five examined were contaminated with bacteria that grew in the broth. There were no capsules in which fluid was contaminated but embryos were not and *vice versa*.

All 35 of the pre-shelled embryos reared in seawater with antibiotics survived 21 days. In contrast, the eight control embryos were all dead by Day 4. By Day 2, one control embryo had expelled all the yolk it contained, and the two control embryos that survived through Day 3 also expelled their yolk between inspection on Day 2 and inspection on Day 3. (See Pechenik *et al.*, 1984 for a description of yolk expulsion.) The other control embryos disintegrated or had yolk protruding from parts of the body other than the mouth.

During the first six days of the experiment with excapsulated embryos, the number of embryos attached to the nurse egg masses changed. For example, on Day 3 no embryos in dish 1 were attached to the nurse egg mass, but on Day 4 two were on the mass, on Day 5 there were no embryos on the mass, and on Day 6 two embryos were again on the mass. These observations indicate that embryos could move off the masses and return later.

Over the 21 days of the experiment with excapsulated

embryos, the embryos in seawater with antibiotics developed shells and eyes. By the end of the experiment, the shells of larger embryos had siphons, and shell lengths ranged from 453 μm to 1192 μm. Along with the 35 normal, yolk-containing embryos, there were 10 runts (embryos with little or no yolk) in the three dishes. These runts also survived the entire 21 days, but they did not differentiate noticeably.

Fluid from the three *Buccinum undatum* egg capsules was axenic. No bacteria were found in fluid from three of the *Thais haemastoma canaliculata* capsules. However, bacteria were found in one capsule. This was an older capsule with embryos ready to emerge; it may have been damaged.

DISCUSSION

Prosobranch egg capsules may provide protection against some predators (Pechenik, 1979; Perron, 1981) and salinity stress (Pechenik, 1982, 1983). Although the capsular fluid is not bacteriostatic, this study indicates that the egg capsules of *Nucella lapillus* provide a bacteria-free environment for developing embryos. In all capsules in which living embryos were found, capsular fluid and embryos were axenic. Death of embryos does not necessarily indicate that capsules are contaminated, suggesting that capsules with dead embryos may retain their impermeability to bacteria.

Generally, dead or moribund embryos of this species turn pink as a response to environmental stress (Pechenik, 1982, 1983), as embryos exposed to deionized water in this study did. However, two of the contaminated capsules contained dead embryos that had retained their creamy yellow color. Excapsulated embryos exposed to 0.45 μm seawater also retained their yellow color, even after death. It is possible that embryos that die from exposure to bacteria do not turn pink, unlike those that are exposed to salinity or temperature stress. Spight (1977) reports that hermit crabs cannot puncture the capsules of the West Coast muricid *Nucella lamellosa*. However, even a failed predation attempt may damage a capsule, allowing bacteria to enter and kill the embryos inside. Further work needs to be done to test this possibility.

While the embryos of some gastropod species can be raised outside their capsule (e.g. *Ilyanassa obsoleta* [Say]; Costello and Henley, 1971), previous attempts to raise pre-shelled embryos of *N. lapillus* have been unsuccessful (Pechenik *et al.*, 1984). In this study, 100% of the pre-shelled embryos survived and developed eyes and shells when reared axenically. This indicates two things: 1) pre-shelled embryos of this species are susceptible to bacteria found in seawater, and 2) the capsular fluid is not necessary for normal development of *N. lapillus* embryos. This second finding supports work done by Pechenik *et al.*, (1984) showing that the fluid from capsules of *N. lapillus* is not necessary for normal growth of developing embryos.

After *N. lapillus* embryos develop shells, they can be reared outside the capsule in non-sterile 0.45 μm filtered seawater (Pechenik *et al.*, 1984). This indicates that embryos loose their susceptibility to bacteria at some time during their development. Further research is needed to determine when *N. lapillus* embryos become resistant to bacteria, and if resistance is associated with development of the shell.

Preliminary work indicates that fluid from egg capsules of the gastropods *Thais haemastoma canaliculata* and *Buccinum undatum* is also axenic. More work needs to be done on other species to determine whether gastropod egg capsule contents are generally axenic. This study indicates that, even when the fluid from egg capsules does not have bacteriostatic properties, egg capsules themselves may protect against bacteria by providing an axenic microenvironment for developing embryos.

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LITERATURE CITED

Bayne, C. J. 1968. Histochemical studies on the egg capsules of eight gastropod molluscs. *Proceedings of the Malacological Society of London* 38:199-212.

Costello, D. P. and C. Henley. 1971. *Methods for Obtaining and Handling Marine Eggs and Embryos*. 2nd edition. Woods Hole, Massachusetts: Marine Biological Laboratory. 247 pp.

Gallardo, C. S. 1979. Developmental pattern and adaptations for reproduction in *Nucella crassilabrum* and other muricacean gastropods. *Biological Bulletin* 157:453-463.

Giese, A. C. and J. S. Pearse. 1974. Introduction and general principles. *In: Reproduction of Marine Invertebrates*. Vol. 1. Acoelomate and Pseudocoelomate Metazoans. A. C. Giese and J. S. Pearse, eds., pp. 1-49. Academic Press, New York.

Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Applied Environmental Microbiology* 33(5):1225-1228.

Langdon, C. J. 1983. Growth studies with bacteria-free oyster (*Crassostrea gigas*) larvae fed on semi-defined artificial diets. *Biological Bulletin* 164:227-235.

Manahan, D. T., J. P. Davis, and G. C. Stephens. 1983. Bacteria-free sea urchin larvae: selective uptake of neutral amino acids from seawater. *Science* 220:204-206.

Pechenik, J. A. 1979. Role of encapsulation in invertebrate life histories. *The American Naturalist* 114(6):859-870.

Pechenik, J. A. 1982. Ability of some gastropod egg capsules to protect against low-salinity stress. *Journal of Experimental Marine Biology and Ecology* 63:195-208.

Pechenik, J. A. 1983. Egg capsules of *Nucella lapillus* (L.) protect against low-salinity stress. *Journal of Experimental Marine Biology and Ecology* 71:165-179.

Pechenik, J. A., S. C. Chang, and A. Lord, 1984. Encapsulated development of the marine prosobranch gastropod *Nucella lapillus*. *Marine Biology* 78:223-229.

Perron, F. E. 1981. The partitioning of reproductive energy between ova and protective capsules in marine gastropods of the genus *Conus*. *The American Naturalist* 118(1):110-118.

Rivest, B. R. 1981. Nurse egg consumption and the uptake of albumen in the embryonic nutrition of marine snails. Ph.D. dissertation, University of Washington. 185 pp.

Spight, T. M. 1975. Factors extending gastropod embryonic development and their selective cost. *Oecologia* 21:1-16.

Spight, T. M. 1977. Do intertidal snails spawn in the right places? *Evolution* 31(3):682-691.

Thorson, G. 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biological Review* 25:1-45.